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SHORT-TERM VARIATIONS OF MICROBIOLOGICAL AND PHYSICOCHEMICAL PARAMETERS IN SUBMERSION WATER OVER A RICE FIELD

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SUMMARY

Diurnal variations of the number of colony-forming bacteria and total bacteria and of some physicochemical parameters (temperature, pH, redox potential, oxygen concentration, carbonates and total alkalinity) have been studied in the flood layer over the rice field. Confidence limits (95 %) around means were evaluated according to accuracy of plate and microscopic counts and to patchiness within the rice field. Bacterial clumping both at the meter and the millimeter scale has been evidenced, and transformation of raw data was necessary to normalize counts. Seven per cent of total bacteria enumerated by epifluorescence microscopy could develop colonies on agar plates. Significant variations of numbers of colony-forming bacteria and bacterial biomass (evaluated to 0.016 mg/l dry-weight) were not correlated with physicochemical parameters within the flood layer, indicating additional influence of grazing and exchange with sediment interface.

KEY-WORDS: Soil, Water, Rice, Bacterial growth; Short-term variations, Physico-chemistry.

INTRODUCTION

Within the delta of Camargue (South of France) rice fields flooded during summer constitute at the same time relay environments for aquatic biocenosis and sources of pollution, for the National Biological Reserve,

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due to pesticide treatments. A multidisciplinary study (hydrology, chemistry, algology, hydrobiology, bacteriology and weed science) of the flood water was therefore undertaken in order to evidence the importance of paddy-fields with respect to both problems. The first aim of the microbiological study was to evaluate short-term and long-term variations of the bacterial biomass during the crop season, and to correlate these fluctuations with physicochemical parameters.

Despite the fact that microorganisms react quickly to changes in their environment, most studies are based on samples taken at weekly or monthly intervals, and short-term investigations are neglected. The present paper reports on evaluation of precision of 2 enumeration methods, patchiness within the water layer over the rice field and short-term variations at different periods of the crop season. Interactions between microbiological and physicochemical parameters are discussed.

MATERIALS AND METHODS

1) *Rice field.*

Experiments were performed during summer 1979 in a paddy-field (100 × 90 m) where flooded rice had been grown each summer for 3 years without pesticide treatment. The laboratory was situated next to the field, and therefore samples could be treated within 10 min after collection. Physicochemical analysis of a mean sample of soil collected prior to fertilization was performed in ORSTOM laboratories of soil science. Basal fertilizer (formula 15,20,10) incorporated to ploughed soil contained 120 kg/ha nitrogen element. Rice variety Cigalon (200 kg) was sown 2 days after flooding on May 11 and the water level was maintained between 10 and 15 cm during all the crop season.

Stratified random sampling was used in the paddy-field to prevent all randomly selected sites from being in one part of the field. Excepting a 10 m border all around, the experimental field was divided into $7 \times 6 = 42$ squares of 12.5 m side. In each square one sampling site was located at random so that minimal distance between 2 sites was 2 m. Thus distance between contiguous sampling sites fluctuated between 2 and 32 m.

2) *Physicochemical determinations.*

All measurements were performed jointly by the staff of the laboratory in the frame of the multidisciplinary study. Illumination was measured using a portable « Guerpillon 490 » luxmeter. Temperatures were recorded with a « Lambrecht » telethermograph at different depths in a fixed station and at differend sites with the temperature probe of an YSI oxymeter. pH and Eh were measured *in situ* with a « Metrohm Herisau E 250 » pH meter. Dissolved oxygen was determined *in situ* using a YSI oxymeter. Total alkalinity and carbonates were evaluated at the laboratory in the mean samples used for bacterial enumerations.

3) *Sample treatment.*

Immediatly after collection, samples taken to the laboratory were diluted if necessary in a sterile salt solution containing NaCl, 25 g; $MgSO_4$, 1 g; KCl, 1 g; distilled water, 1,000 ml.

INT = 2-(p-iodophenyl)-3(p-nitrophenyl)-5-phenyl tetrazolium chloride.

4) *Spread plate enumerations.*

Colony-forming bacteria were enumerated by spreading 0.2 ml of the appropriate dilution on 10 cm Petri dishes containing 20 ml of the following medium autoclaved at 110° C for 20 min: « Difco » Bacto nutrient agar, 23 g; « Difco » yeast extract, 1 g; diluting salt solution, 1,000 ml. Colonies were enumerated after 5, 10 and 20 days of incubation at 30° C.

5) *Evaluation of spread plate count accuracy.*

Variance associated to subsampling within the final dilution was evaluated in 2 different experimental procedures: 30 individual pipettings of 0.2 ml or 6 groups of 5 plates spread from 1 ml pipettings.

Variance associated to dilution was determined by comparing means of 5 repetitions from 24 series of dilutions 10^{-5} of the same water sample.

Patchiness within the rice field was evaluated by sampling 36 randomized sites as discussed above. From each sample (60 ml collected in sterile vials from the medium depth of the water layer) serial dilution and 5 replica of the 10^{-5} dilution were prepared.

6) *Influence of delay between sampling and spreading.*

From the same water sample, serial dilutions were prepared immediatly after collection and after 2, 5 and 10 h of conservation at room temperature. Five replica were plated from each final dilution.

7) *Diurnal variations of the number of colony-forming bacteria.*

Colony-forming bacteria were enumerated at 3-h intervals during 31 h in the water layer, 5 weeks after sowing (June, 12) and 9 days later (June, 21).

8) *Counts by epifluorescence microscopy.*

Total number of bacteria and respiring microorganisms were enumerated by the method of Zimmermann *et al.* [39]. A 1 ml amount of 0.2 % aqueous solution of INT dye was added to 10 ml subsamples of freshly collected water and the reaction stopped with 0.1 ml of 37 % formaldehyde after 20 min incubation in the dark. A 2 to 5 ml amount of eventually diluted fixed sample was filtered then stained by contact during 2 min with 1 ml of an acridine orange solution (1/10,000 in 6.6 mM phosphate buffer, pH 6.7). Mounted filters were examined by epifluorescence microscopy (Zeiss standard microscope fitted with an IV-FL epicondenser, a 50 W HBO halogen lamp, a 455-490 band-pass filter, a 510 beam splitter and an LP-500 barrier filter). The total number of bacteria and the proportion of respiring bacteria were determined within areas delimited by a 10×10 granulated eyepiece. Between 100 and 500 bacteria were counted in each area.

9) *Evaluation of microscopic count precision.*

Variance due to heterogeneity within a subsample (*i. e.* within the same filter) was evaluated by counting 34 different areas of 1 filter.

Patchiness within water samples was determined by preparing 31 different filters from the same water sample.

Patchiness in the distribution of total bacteria within the flood layer was evaluated by sampling 40 randomized sites in the paddy-field as discussed above.

10) *Diurnal variations of physicochemical parameters and number of total bacteria.*

Total number of bacteria were evaluated each 3 h during 33 h in the water layer over the paddy-field at the flowering stage of rice (« closed medium » with

vegetation covering the water) and in an enclosure (10 × 3 m) manually cleaned of vegetation reserved in the field. Statistical analysis were performed using an HP-97 programmable calculator.

RESULTS

1) *Physicochemical parameters in the paddy-soil and the water layer*

Some physicochemical parameters of the plough layer soil are reported in table I. The experimental field is a silty organic soil with a nitrogen content of 10 mmole N/100 g dry-weight.

TABLE I. — **Some physicochemical parameters of the plough layer soil.**

Apparent density	1.38
Actual density	2.63
Porosity (%)	47.5
Clay, 0-2 μm (%)	20.0
Silt, 2-20 μm (%)	39.0
Silt, 20-50 μm (%)	22.0
Sand, 50-200 μm (%)	11.0
Sand, 200-2,000 μm (%)	3.0
Total organic matter (% d. w.)	3.7
Total nitrogen (% d. w.)	0.14

Analysis of a mean sample after mixing of 30 samples of 100 g.

At the early stages of the rice crop, light is totally transmitted to the water layer (« open medium »), but only 3 to 25 % are still recorded during the second part of the culture (« closed medium »). Consequently, the average diurnal thermal amplitude in the water layer was 18° C in the conditions of the « open medium » (May, and enclosure devoided of vegetation), but only 3° C in the conditions of « closed medium » (August).

Long-term variations of dissolved oxygen concentration are correlated to the development of photosynthetic planktonic organisms; values up to 20 mg O_2/l (corresponding to 245 % saturation) were recorded in day-time during May and June. In August, oxygen decreased to only 9 mg/l (110 % saturation) in day-time and 1 mg/l during night. One week after flooding, the redox potential at 1 cm depth in the soil was decreased to — 220 mV, but remained around 300 mV in the water layer.

Diurnal variations of pH, correlated to the complex carbonic acid system, were around values of about 9 in May-June (« open medium ») and 7.5 in August (« closed medium »). Total alkalinity increased from 150 up to 250 mg/l in May and August, respectively, mainly in the form of hydrogen carbonates.

Nitrate and nitrite could only be detected in the first 10 days of sub-

mersion, after which ammonium was the only nitrogen form with an average concentration of 0.15 mg/l in the water layer.

2) *Precision of spread plate enumerations*

Experimental design for evaluating variances associated to the different levels of analysis is represented in figure 1.

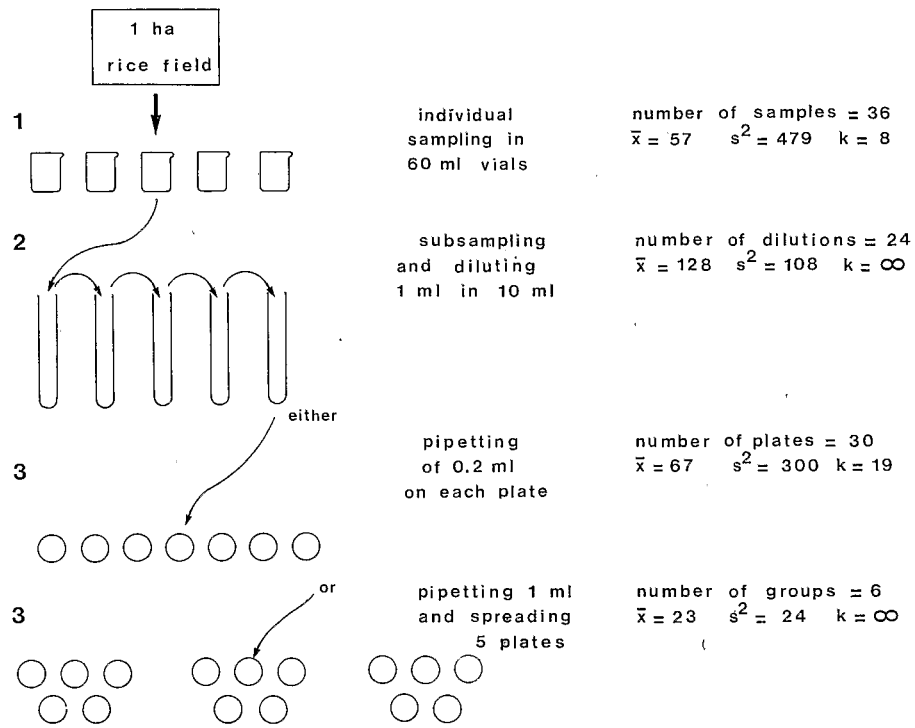

FIG. 1. — *Experimental design for evaluation of accuracy of spread plate counts and patchiness in the distribution of colony-forming bacteria.*

a) *Subsampling within the last dilution.*

Agreement of distribution of 30 counts corresponding to individual pipettings of 0.2 ml from the same final dilution to a Poisson series was tested with the χ^2. A valuable approximation of χ^2 is the expression I(n−1), where I is the variance to mean ratio index of dispersion : $I = \frac{s^2}{\bar{x}}$.

Therefore, χ^2 can be approximated to $I(n-1) = \frac{s^2(n-1)}{\bar{x}}$.

The calculated χ^2 value of 3,895 was well above the 5 % point of

42.56 for 29 degrees of freedom, and the hypothesis of randomness is disproved.

The negative binomial distribution is often a suitable mathematical model for clumped or aggregated populations where the variance σ^2 (estimated by s^2) is greater than the mean μ (estimated by $\bar{x}$). The probability series is then given by the expansion of $(q - p)^{-k}$, where $p = \frac{\mu}{k}$ and $q = 1 + p$.

The statistic parameter k is related to the spatial distribution of the population, and as variance $\sigma^2 = kpq = \mu + \frac{\mu^2}{k}$, therefore $k = \frac{\mu^2}{\sigma^2 - \mu}$.

The estimate value $\left(\hat{k} = \frac{\bar{x}^2}{s^2 - \bar{x}}\right)$ of k is then a measure of the clumping of individuals.

The negative binomial distribution is asymetrical for a large range of arithmetic means when k is small (less than 5), but approaches normality when k increases ($k > 10$) and the mean is large ($\bar{x} > 10$).

In the negative binomial distribution the variance of the sample is not independant of the mean, and statistic tests associated with the normal law cannot be applied without the risk of considerable errors. A suitable transformation of data is therefore necessary. According to Taylor [36], the variance (σ^2) of a population is proportional to a fractional power of the arithmetic mean (μ), $\sigma^2 = a\mu^b$. Therefore, $\log \sigma^2 = \log a + b \log \mu$, a and b being population parameters. If μ and σ^2 are estimated by the statistics $\bar{x}$ and s^2, then $\log s^2 = \log a + b \log \bar{x}$. If the original counts x are replaced by x^p when $p = 1 - b/2$, then the methods associated to the normal distribution are applicable to the transformed data x^p. Parameter b was estimated by the regression of $\log s^2$ on $\log \bar{x}$, either graphically on a double-log scale or by a modified programme on a HP 97 calculator, from 89 groups of 5 plates corresponding to pipettings of 1 ml distributed by 0.2 ml on 5 Petri dishes. From the values $\log s^2 = -0.14 + 1.24 \log \bar{x}$, with $r = 0.69$ (significant at the 1 % level) the transformation

$$y = x^{1 - \frac{1.24}{2}} = x^{0,38}$$

was calculated and applied to the data. It was then checked either graphically or by the calculator programme that the means of transformed count $\bar{y}$ were independant of the variances.

After the transformation $y = x^{0,38}$ the F test for one-way analysis of variance can be applied to 6 groups of 5 plates from the same final dilution. As the calculated value $F^5_{24} = 2.17$ is smaller than the tabulated, the null hypothesis that the 6 groups come from normally-distributed populations with the same mean and variance cannot be rejected at the 95 % probability level. Therefore, counts from 5 plates were trans-

formed, and 95 % confidence limits around the transformed means $\bar{y}$ were

$$\bar{y} \pm t\sqrt{\frac{s_y^2}{n}} = \bar{y} \pm 2.77\sqrt{\frac{s_y^2}{5}}$$

s_y^2 being the variance of transformed counts [10].

Then the derived means ($\bar{x}'$) were calculated by the back transformation $\bar{x}' = e^{\frac{\text{Ln}\,\bar{y}}{0.38}}$ and the relative errors in terms of percentage confidence limits of the derived means $\bar{x}'$ were estimated to $\pm$ 12 % for 1 group of 5 plates and $\pm$ 7 % for 2 groups of 5 plates from the same final dilution.

b) *Subsampling and diluting from the water sample.*

Variance associated to subsampling 1 ml in the water sample and serial dilution to 10^{-5} was calculated from 24 series of dilutions prepared from the same sample. After transformation $y = x^{0.38}$, the calculated value of $F_{84}^{23} = 1.35$ is smaller than the tabulated, and agreement with a Poisson series cannot be rejected at the 95 % probability level. As $\bar{x}$ was higher than 30, the Poisson series was approximated to a Gaussian distribution. Therefore, it can be assumed that 95 % of estimates ($\bar{x}'$) of the true mean (μ) lie into the interval $\mu \pm 1.96\,\sigma$ (Gaussian distribution), with $\mu = \sigma^2$ (Poisson distribution).

As a consequence, errors as a percentage of the unique derived mean $\bar{x}'$ from 5 plates of the same final dilution can be approximated by $\frac{1.96 \times 100}{\sqrt{\bar{x}'}}$ if $\bar{x}' > 100$, thus to about $\pm$ 20 % if 100 colonies are enumerated on each plate.

It must be noticed that error is proportional to $\frac{1}{\sqrt{\bar{x}'}}$.

Therefore in the serial dilution process errors associated to the first dilutions (10^{-1}) were negligible as the number of organisms present was at least in the order of 10^5.

c) *Patchiness within the rice field.*

Sixty-ml samples were collected from 36 randomized sites as discussed above, and derived mean numbers of colony-forming bacteria were calculated after the transformation $y = x^{0.38}$. Variance $s^2 = 479$ was significantly higher than the mean $\bar{x}$ of the 36 derived means. The calculated χ^2 value of 294 was well above the tabulated, and thus agreement with a Poisson distribution was rejected.

Agreement with a negative binomial distribution was tested by the U test comparing the estimated variance (s^2) and the expected variance in the negative binomial series $\left(\bar{x} - \frac{\bar{x}^2}{k}\right)$.

$U = s^2 - \left(\bar{x} - \frac{\overline{x^2}}{k}\right)$ has an expected value of zero for perfect agreement, which is accepted if the calculated value differs from zero by less than its standard error. The standard errors of U are given in tables [9].

Calculated value of parameter U was 1, which is considerably less than the standard error. Therefore, agreement of the distribution of the 36 derived means calculated from the different sites in the flooded rice field with a negative binomial distribution cannot be rejected at the 95 % probability level.

The logarithmic transformation was applied to normalize the derived means, and validity of transformation was checked graphically on « probit » paper; 95 % confidence limits around the derived mean were then calculated using a HP-97 program according to Roger *et al.* [33]. Values of 12 and 20 % of the derived mean were calculated for 36 and 15 samples, respectively.

The choice of an experimental design for enumeration of colony-forming bacteria was made by comparing total variance of the process and cost which must both be minimized. Total variance is equal to

$$s_t^2 = \frac{s_1^2}{n_1} + \frac{s_2^2}{n_1 \times n_2} + \frac{s_3^2}{n_1 \times n_2 \times n_3}$$

if s_1^2 = variance between individual samples and n_1 = number of samples, s_2^2 = variance between subsamples and serial dilutions and n_2 = number of serial dilutions, s_3^2 = variance between groups of 5 plates from the last dilution and n_3 = number of groups of 5 plates. Maximal total variance is for $n_1 = n_2 = n_3 = 1$, *e. g.* $s_t^2 = 700$ if $\bar{x} = 100$.

In table II are reported the total variances and corresponding numbers of plates to be counted. It was clear that for the same number of plates the best experimental design would be to treat individually 15 samples and to spread only 1 plate from each of the final dilutions thus obtained, but the cost in time of this process was by far too important and would not allow samplings at 3-h intervals.

TABLE II. — **Diminution of total variance associated to spread plate enumerations as a function of the experimental design.**

Nb of samples n_1	Nb of serial dilutions n_2	Nb of groups of 5 plates n_3	Total nb of plates N	Total variance s_t^2	Diminution from s_t^2 max. (%)
1	1	1	5	700	0 %
1	1	15	75	610	12 %
1	15	1	75	606	13 %
15	1	1	75	233	66 %

If 15 samples were mixed to obtain a mean sample of 15×60 ml$=900$ ml, subsampling 1 ml out of the mean sample corresponded to an additional step of dilution 1/15 at the first dilution level. As discussed above, the relative error associated to the first dilution was negligible due to the number of organisms present.

From all considerations above, the following experimental design was chosen to evaluate the number of colony-forming bacteria in the water layer over the rice field:

— mixing 15 samples of 60 ml from randomized sites corresponded to a relative error of $\pm$ 20 %;

— subsampling 1 ml and diluting generally to 10^{-3} corresponded to a relative error of $\pm$ 20 %;

— spreading 2 series of 5 Petri dishes from the last dilution corresponded to a relative error of $\pm$ 7 %.

Total relative error was thus estimated to $\pm$ 47 % of the derived mean when about 100 colonies are enumerated on each plate.

3) *Influence of delay between sampling and spreading*

One-way analysis of variance was applied to transformed counts ($y = x^{0.38}$) from serial dilutions of a water sample prepared immediately after collection and after 2, 5 and 10 h, respectively. As the calculated $F^3_{15} = 1.75$ is less than the tabulated value, the differences between sample means were not significant. After 24 h at the laboratory temperature, the number of colony-forming bacteria increased significantly ($F^4_{44} = 5.10$) even at the 1 % level.

4) *Diurnal variations of the number of colony-forming bacteria*

In figure 2A are represented variations observed in June 12 and 13. Important fluctuations with a 3-fold increase were recorded, and the peak at 03.00 pm in June 12 corresponded to the multiplication of a unique species representing less than 5 % of the colonies in the morning samples and more than 50 % 3 h later. A decrease from 3.2×10^5 to 0.8×10^5/ml in 2 h was observed just after solar noon. Although counts at 24 h interval are not similar, the number of colony-forming bacteria was found to decrease significantly between 03.00 am (night) and 08.00 am (early morning) both in June 12 and 13.

As shown in figure 2B, no important variation of the number of colony-forming bacteria was recorded in June 21 and 22; a general increase was only detected during the 28 h of the experiment.

5) *Precision of microscopic counts*

Experimental design for evaluating variances associated to the different levels of analysis is represented in figure 3.

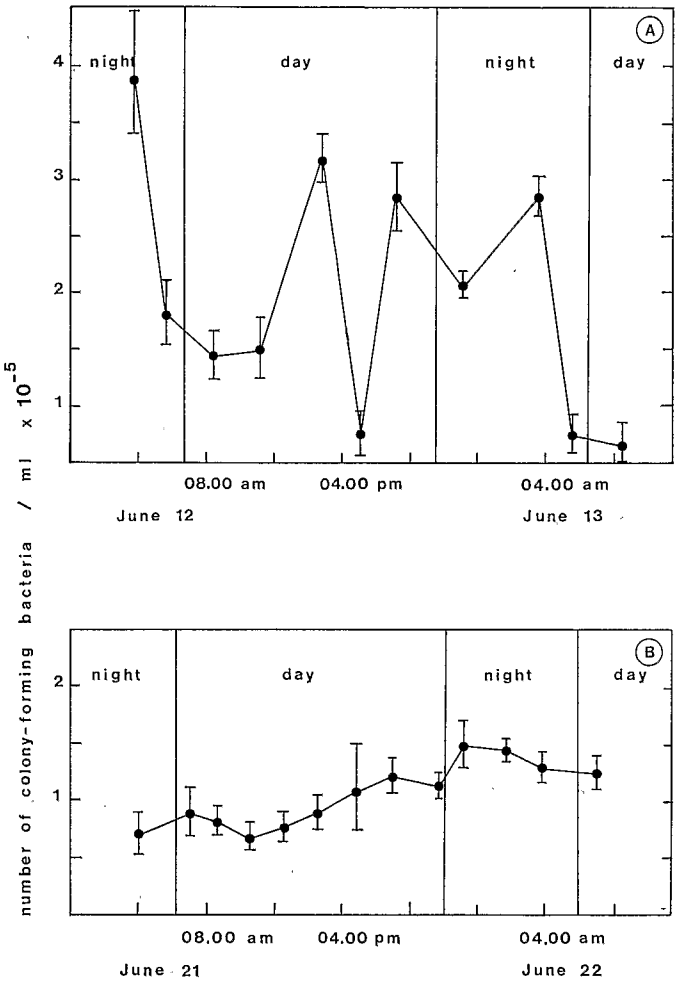

FIG. 2. — *Short-term variations of number concentration of colony-forming bacteria in the flood layer.*

Means and 95 % confidence limits are indicated.
A : June 12 and 13, 1979.
B : June 21 and 22, 1979.

a) *Variance associated to heterogeneity within subsamples.*

Microscopic examination of membranes supporting 3 to 5 ml subsamples pipetted after gentle agitation of a 60 ml sample shows 3 different types of association between bacteria.

In all areas are seen isolated bacteria, easy to be counted if their total number does not exceed 500/area. In about 1/3 of areas are small aggregates of 5 to 50 bacteria either associated in « colonies » or bound to small particles. Exceptionally (*e. g.* in less than 1/50 of areas) are bigger organic

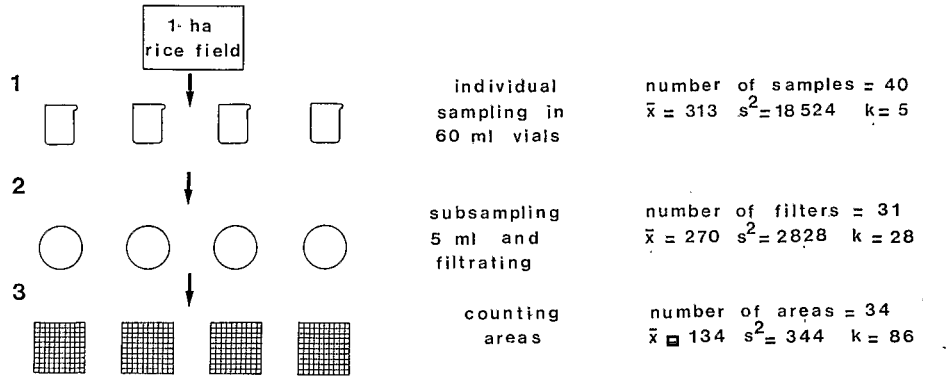

FIG. 3. — *Experimental design for evaluation of accuracy of epifluorescence microscopic counts and patchiness in the distribution of total bacteria.*

particles (average size 100 μm) supporting several hundreds of bacteria.

The proportion of aggregated bacteria was around 2.5 % of total bacteria in all water samples from the paddy-field, and therefore aggregates were not taken into account in the determination of accuracy of microscopic counts.

From 34 areas enumerated in the same filter, 11 contained 1 small aggregate (10 to 60 bacteria) and 1 contained 2 aggregates of 20 and 30 bacteria. The proportion of aggregated bacteria was determined as a microbiological parameter, but calculations were made on the total number. Therefore $n = 34$, $\bar{x} = 134$, $s^2 = 344$, $k = 86$. The calculated χ^2 value of 85 is well above the tabulated and agreement with a Poisson series was rejected. Statistic parameter U was less than its standard error, and therefore agreement with a negative binomial distribution was not rejected at the 95 % level.

The validity of the logarithmic transformation $y = \log x$ was checked graphically on « probit » paper; 95 % confidence limits around derived means as a function of areas and bacteria enumerated were calculated using the HP-97 program as discussed above. It was clear that relative errors were minimized by increasing the number of areas counted rather than the density of cells on the filters. Values of 11 and 8 % of derived means were calculated for 10 areas counted supporting 50 and 350 bacteria, respectively.

b) *Patchiness within water samples.*

Thirty-one subsamples of 5 ml were filtered from the same water sample and derived means calculated after the logarithmic transformation of counts from 10 areas on each filter: $n=31$, $\bar{x}=261$, $s^2=3{,}009$, $k=25$. The calculated χ^2 value was well above the tabulated and thus agreement with a Poisson series was rejected. On the contrary, the n means $\bar{y}$ of

transformed data are distributed according to a Poisson series of mean

$$\frac{\Sigma\bar{y}}{n} \text{ and } s^2 = \frac{s_1^2 + s_2^2 + \ldots + s_n^2}{n^2}$$

Thus percentage 95 % confidence limits around the derived mean were evaluated to $\pm$ 40 % and $\pm$ 28 % when 1 or 2 subsamples were, respectively, filtered from a water sample and 10 areas enumerated on each filter.

c) *Patchiness within the rice field.*

Derived means calculated from 10 counts on 40 filters corresponding to randomized samples were calculated: $n=40$, $\bar{x}=305$, $s^2=17.466$, $k=5$. Agreement with a Poisson series was disproved by the χ^2 test, thus showing non-randomness of the distribution of 60 ml samples within the water layer over the rice field.

As discussed above, the means of transformed counts were randomly distributed. Calculated percentage 95 % confidence limits around derived means as a function of the number of samples and bacteria when 10 areas were enumerated are reported in table III.

TABLE III. — **Percentage 95 % confidence limits of derived means of microscopic counts as a function of the experimental design.**

Nb of samples	Average count from 10 areas	Total nb of bacteria enumerated	Percentage 95 % confidence limits of mean
1	350	3,500	$\pm$ 40 %
15	50	7,500	$\pm$ 26 %
15	150	22,500	$\pm$ 25 %
15	350	52,500	$\pm$ 25 %
40	50	20,000	$\pm$ 15 %
40	150	60,000	$\pm$ 14 %
40	350	140,000	$\pm$ 14 %

As for plate counts, it was evident that increasing the number of samples was more efficient in order to decrease 95 % confidence limits. About 15 individually treated samples were necessary to obtain a 25 % error, but the cost in time to prepare and filtrate 15 samples was too important. Therefore a mean sample was obtained by mixing 15 samples of 60 ml, and we have stated above that the corresponding additional error was negligible.

From all considerations above, the following experimental design was chosen to evaluate number of total bacteria in the water layer over the rice field:

— mixing 15 samples of 60 ml from randomized sites corresponded to a relative error of $\pm$ 25 %;

— filtrating 2 subsamples of 5 ml corresponded to $\pm$ 28 %;

— counting isolated bacteria in 10 microscopic areas corresponded to ± 8 %;

— evaluating the number of bacteria forming small colonies or aggregated onto particles in a total of 30 areas; for biomass determinations, aggregated bacteria were therefore taken into account.

Total relative error was thus estimated to ± 61 % of the derived mean when about 250 bacteria were enumerated in each area.

6) *Diurnal variations of physicochemical parameters and number of total bacteria*

In figures 4 and 5 are reported variations of some physicochemical parameters and number of total bacteria in the enclosure devoided of vegetation and in the rice field, respectively.

During the 33 h of experiment in August 21 and 22, variation amplitudes of physicochemical parameters were more important in the enclosure than under vegetation in the rice field. As discussed above, pH was mainly influenced by the shift of the carbonic acid equilibrium, the removal of free CO_2 by photosynthesis increasing pH during day-time. Dissolved oxygen increased from 2.3 mg/l (29 % saturation) during night to 9 mg/l (110 %) and 18 mg/l (220 %) at solar midday in the rice field and the enclosure, respectively. Values of redox potential, evaluated after correction due to pH, fluctuated between 275 to 355 mV and 290 to 335 mV in the enclosure and the paddy-field, respectively. From figures 4 and 5 it can be seen that redox potential fluctuations were not linked to dissolved oxygen concentrations. This statement was confirmed using the HP 97 program to test the relation $Eh = a + b\,LN[CO_2]$. The calculated correlation coefficient $r = 0.2$ is well below the tabulated value and correlation was not significant.

The « buffering effect » of vegetation was also noticed on temperature which remained constant at the bottom layer, but fluctuated with an amplitude of 6 and 10° C in the rice field and the enclosure, respectively.

The ratio of colony-forming bacteria to total bacteria was found to be 7 % in a mean sample of the water layer in August. Although variations of this ratio corresponding to changes in the qualitative composition of the microflora might be possible, it was physically impossible for us to evaluate this proportion in all samples.

On the contrary, percentages of « respiring » bacteria exhibiting formazan granules after INT treatment were determined in each sample but did not significantly fluctuate during our experiment. Only 5 % of bacteria developed visible formazan spots under transmitted bright-field illumination.

When examining the filters by epifluorescence illumination, about 57 % of bacteria exhibited an orange fluorescence and 43 % a green fluorescence, and this proportion did not change significantly with time.

Mean values and 95 % confidence limits of number concentration of total bacteria are reported in figures 4 and 5. Both in enclosure and rice

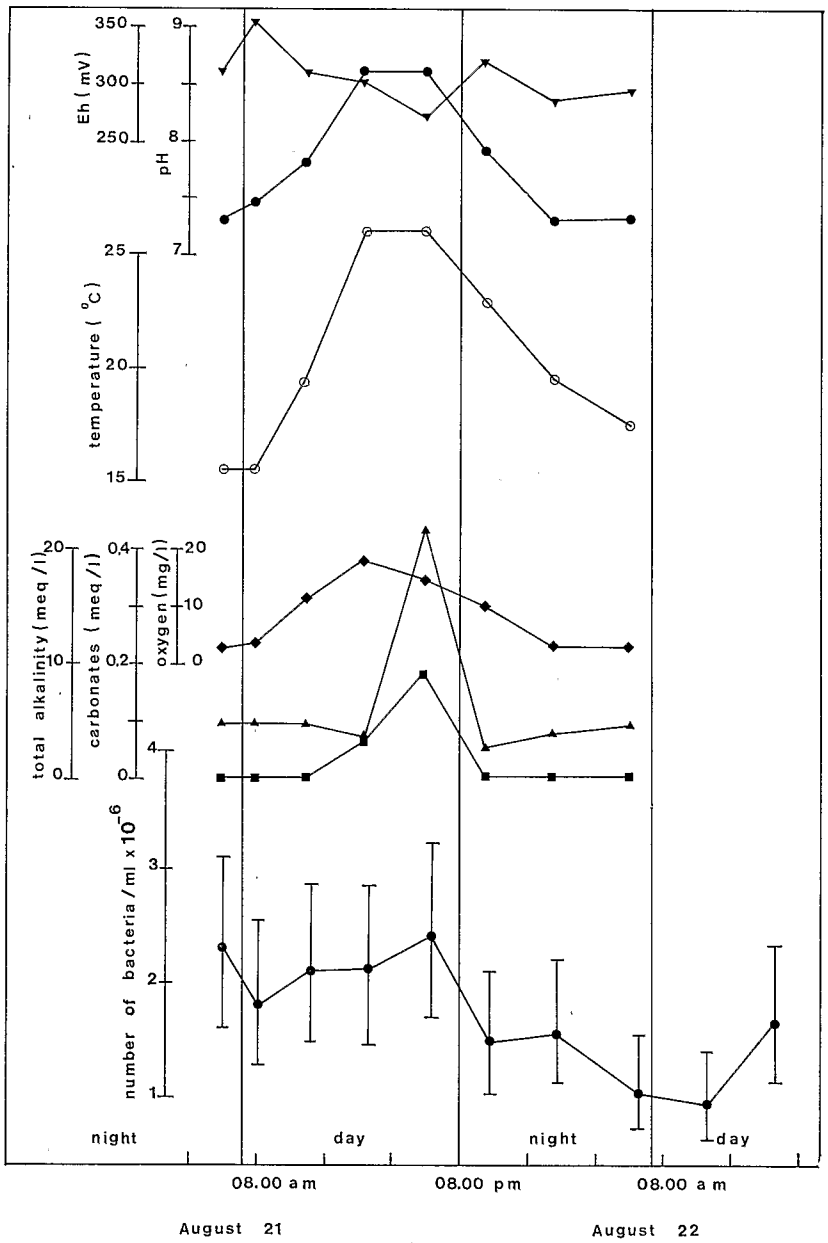

FIG. 4. — *Short-term variations of physicochemical parameters and number concentration of total bacteria in the enclosure devoided of vegetation, August 21 and 22, 1979.*

Eh (▼) pH (●), temperature medium depth (○), total alkalinity (▲), carbonates (■), dissolved oxygen (♦), means and 95 % confidence limits of number concentration of total bacteria (lower curve) (●).

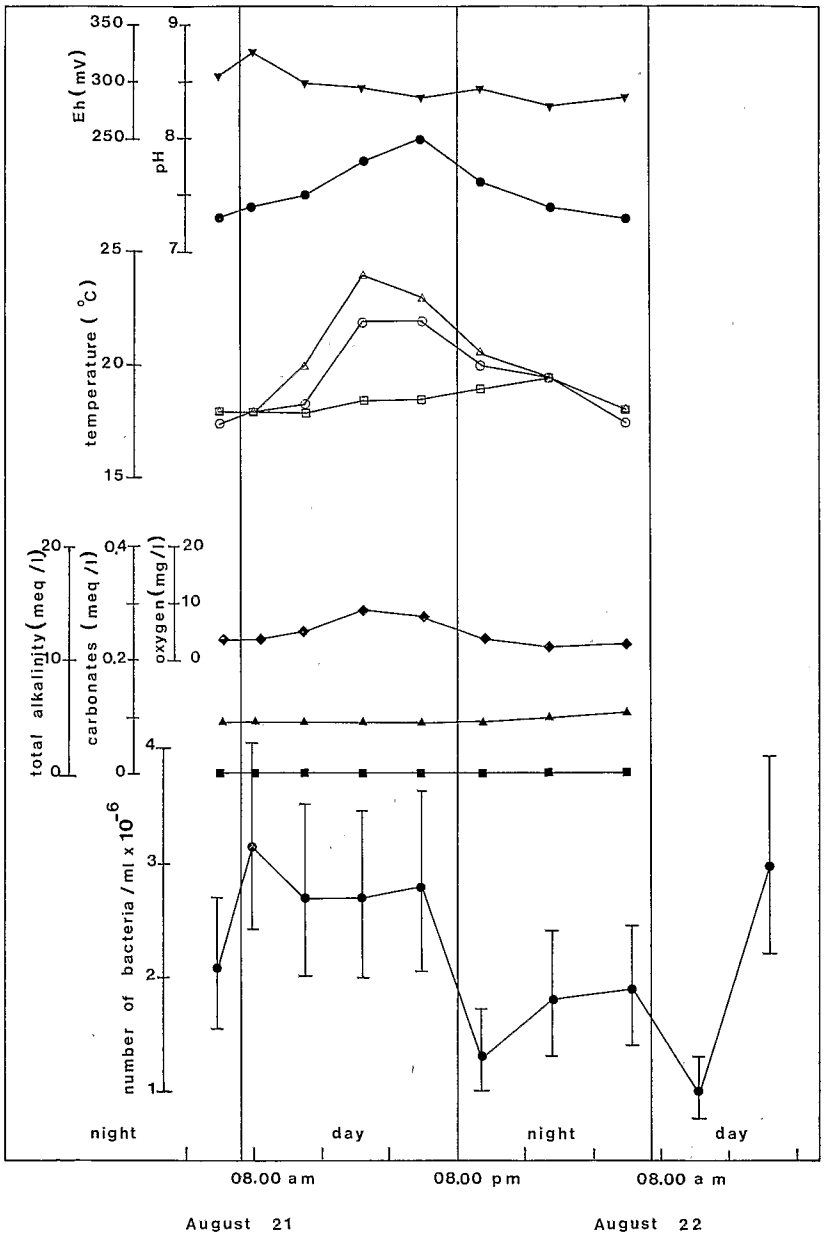

FIG. 5. — *Short-term variations of physicochemical parameters and number concentration of total bacteria in the rice field, August 21 and 22, 1979.*

Eh (▼), pH (●), surface temperature (△), middle depth temperature (○), bottom temperature (□), total alkalinity (▲), carbonates (■), dissolved oxygen (♦), means and 95 % confidence limits of number concentration of total bacteria (lower curve) (●).

field, number of total bacteria in the water layer fluctuated between 1×10^6 to 2.3×10^6/ml (enclosure) and 1×10^6 to 3.2×10^6/ml (rice field). Fluctuations were not significant in the enclosure.

In the rice field, on the contrary, significant differences were noticed with a 2-fold decrease between 06.00 pm (2.8×10^6/ml) and 09.30 pm (1.3×10^6/ml) and 02.30 pm (3×10^6/ml).

The average cell volume calculated was 0.033 nm^3. Assuming that the density of a bacterium is 1.1 with a dry-weight to wet-weight ratio of 0.23 [24], then given 10^6 and 3×10^6 bacteria per ml, the bacterial biomass fluctuated in the flood layer between 0.008 and 0.025 mg/l dry-weight.

DISCUSSION

Seasonal and diurnal variations of the physicochemical parameters of the water layer over the rice field have been well documented [13, 28], and the values reported in the present paper are in agreement with previous studies [31, 32]. Influence of these parameters on the bacterial microflora is discussed further.

Spread plate enumerations have been found to be of smaller precision than pour plate enumerations, but a loss of viability of some bacteria coming into contact with hot agar has been evidenced, resulting in higher average counts in spread plates [35]. As a study of the qualitative composition of the bacterial population within the flood layer was to be performed, it seemed preferable to collect more colonies by spread plates.

Variances associated with each step of the spread plate procedure has been evaluated in previous studies [22, 29, 35]. Although patchiness within water samples or subsamples was observed in some experiments [22] our data are in agreement with previous statement that bacterial clumping on the scale of millimeter could be suppressed by vigorous shaking of bottles and test tubes used for dilutions [23].

Patchiness on the scale of meter has been demonstrated in soils [33], lakes [18, 29], estuarine sediments [2] and sea-waters [3, 22], and the logarithmic transformation is generally suitable to normalize counts. The biological signification of the distribution pattern of organisms in natural environments has been discussed [6, 12], and departure from a Poisson series is attributed both to heterogenous distribution of environmental factors and to clumping of individuals in colonies [9].

As the size of the sampling unit (60 ml) was found to be much larger than the average clumps of bacteria, the negative binomial distribution of 60 ml samples spaced between 2 and 32 m apart might be attributed to patchiness in the distribution of environmental factors within the rice field at the 10 m scale. Similarly Palmer *et al.* [29] have noticed in lakes that although dispersion on the 1 m scale was relatively random, water samples spaced up to 20 m apart were commonly found in different patches.

Keepind in mind that the general purpose of the study was to evaluate

fluctuations of bacterial biomass, 60-ml samples randomly collected about 10 m apart were therefore convenient to take into account patchiness within the rice field.

The practical scheme chosen in order to minimize both total variance and cost (in time and price) corresponded to percentage 95 % confidence limits of $\pm$ 35 % around derived means when about 100 colonies were enumerated on each plate.

Together with Kaper *et al.* [22], we have found that counts of water samples stored at room temperature did not evoluate significantly during 10 h, but increased after 24 h of delay. As the whole experimental procedure from sampling to plating was achieved within 1.5 h, it may be assumed that this delay did not influence the bacterial composition in the samples.

Although observed at only 10 days of interval, the diurnal variations of number of colony-forming bacteria recorded in June (« open medium ») were significantly different. Therefore it may be stated that during this period physicochemical parameters fluctuating with the day-night alternance (*e. i.* illumination, temperature, dissolved oxygen concentration, pH, etc.) were not of prime importance. A dynamic equilibrium has been evidenced in marine environment between both bacterial and plankton populations [25], but the release of organic compounds by primary producers and their consumption by heterotrophic organisms may follow a distinct rhythm [25, 27].

The most striking observation was the rapid multiplication of a bacterial species between 11.45 am and 02.45 pm in June 12. The doubling time of this dominating species calculated from our data is approximately 45 min; an anormaly short time in natural environment where generation times of several hours have been reported [14]. Even more difficult to explain was the significant decrease of the number of colony-forming bacteria from 3.2×10^5 to 0.8×10^5/ml in 2 h, followed by an increase back to high values 2 h later. As the bacterial diversity increased simultaneously, the rapid fluctuations observed were more likely to be under the dependence of the steady state equilibrium between settling and release from interface than of growth and predation, although contents in suspended particles were not significantly different in the samples. In the condition of « open medium » with sparce vegetation this assumption seems reasonable. Therefore the decrease in bacterial diversity corresponding to the increase of number of colony-forming bacteria at 02.45 pm in June 12 could be explained by a sudden perturbation in the water layer, as noticed previously in marine environment [25]. The dominance of a single species on plate counts has also been reported in freshwater lakes during late autumn period of turbulence causing the breakdown of thermal stratification and overtum of the tarn [18]. As a consequence, further experiments should be undertaken in the rice field to check the possibility that during the first part of the crop season bacterial population in the flood layer is mainly influenced by exchanges through interface with the microflora of the sediment.

Since its description by Francisco *et al.* [11] the acridine orange epifluorescence technique has been used for counting bacteria in various environments, and different improvements have been suggested among which are storage of samples [7], use of « Nuclepore » polycarbonate filters [4, 16], dying membranes [20], combination with autoradiography [26] or INT incubation [39].

The first problem arising in microscopic counts is the difficulty to distinguish bacteria from non-living particles. The acridine orange technique has been shown to minimize this problem of subjectivity [4, 20] which anyhow decreases with experience.

The calculated 2.5 % proportion of particle-bound and aggregated bacteria in the flood layer over the rice field was significantly lower than that evaluated in sea-water where proportions of 15 to 93 % have been recorded depending on the depth of the sample [3, 15]. Although an enzymatic treatment has been suggested [5] vigorous agitation of water samples was found sufficient to suppress clumping of bacteria on detritus particles [23]. In the frame of the multidisciplinary study of the rice field, it was assumed that aggregation could be a valuable parameter; therefore water samples were gently agitated prior to subsampling, resulting in the log-normal distribution of counts from different areas on the same filter. Unevent distribution of bacteria was not an artefact of filtration as volumes of about 5 ml were filtered after INT incubation [20] .

As for plate counts, non-randomness was also evidenced between microscopic counts from 40 sampling sites in the rice field, resulting in the poor precision of enumerations unlike previous experiments in other environments [19].

Using pure cultures, general agreement between plate counts and microscopic counts by epifluorescence has been demonstrated [20]. In natural environments, on the contrary, only 0.01 to 2 % of total bacteria were found to form colonies on agar plates [8, 21]. Although microscopic counts are more realistic, plate counts are necessary for isolation of bacterial strains and qualitative studies of microbial populations. Both methods were therefore simultaneously evaluated and used in the rice field. The proportion of 7 % of colony-forming bacteria we found in the water layer over the rice field was slightly higher than values recorded in natural waters.

We found a good agreement of the proportion of 5 % respiring bacteria evidenced by formazan spots in the flood layer with percentages ranging from 5 to 36 % in freshwater lakes or tidal water over a salt marsh [15, 39]. The proportion we found is small, but as previously noticed [39] average biomass of respiring bacteria was significantly higher than that of total bacteria. As a consequence, proportion calculated to the total biomass might in fact exceed by far 5 %. However, our data are in agreement with the previous observations that low percentages of detectable respiring cells correlate with low percentages of particle-bound cells [15].

The theoretical difference between green fluorescence, characteristic of double-stranded DNA and orange fluorescence of RNA [38] has been

proposed to distinguish actively growing bacteria which fluorescence orange-red from inactive bacteria which fluoresce green. However, several observations have shown that validity of this distinction is highly doubtful, as the metachromatic effect was found to be influenced by acridine orange concentration [16, 30].

As a consequence no attention should be paid to the stable proportion between orange and green fluorescent bacteria calculated in the flood layer.

The average bacterial diameter of 0.4 nm, thus corresponding to a bacterial volume of 0.003 nm^3 for cocci, was in agreement with previous studies demonstrating that most of bacteria in sea-waters, freshwaters, sediments and soils are very small cocci with average diameters of 0.5 nm or less [4, 10, 14, 16, 37]. However, 0.2 nm pores of « Nuclepore » polycarbonate membranes have been found suitable to retain most of the bacteria since loss of biomass was negligible as compared to 0.1 nm pores.

The bacterial biomass fluctuated between 0.008 and 0.025 mg/l (dry-weight). At the same time biomass of phytoplankton was evaluated to 0.5 mg/l (A. Vaquer, personal communication), *i. e.* 20 times more at least.

Significative fluctuations of the bacterial biomass recorded during the course of our experiments were not correlated to variations of physico-chemical parameters in the water layer. As discussed previously [34], short-term changes in bacterial populations may not necessarily be in response to a single variable. The present data are too sparse to evidence significant correlations which should take into account the grazing effect by zooplankton.

As shown in figures 4 and 5, variations of the redox potential could not be explained by variations of oxygen concentration resulting from balance between production and respiration. In fact values of the redox potential corresponding to 30 % saturation were, respectively, 280 and 50 mV in phosphate buffer and in a growing bacterial culture [17]. As noticed previously, redox potential in the flood layer was low as compared to oxygen pressures [31, 32].

During night, oxygen concentration remained constant in the water layer in August 21 and 22, with representative values of 2.3 mg/l corresponding to 29 % saturation. At this steady state, a mutual relationship among the specific rate of oxygen uptake (*i. e.* respiration) Q_{O_2}, the cell density X and the dissolved oxygen concentration in the medium $\overline{C}$ is:

$$\overline{C} = C^* - \frac{Q_{O_2} X}{K_l a}$$

when C* is the dissolved oxygen concentration which is in equilibrium with partial pressure in air (*i. e.* 0.2 atm), and $K_l a$ the volumetric oxygen transfer coefficient (h^{-1}).

Various physical and chemical factors affect the values of $K_l a$ (ratio between interface and volume, agitation of liquid and air, temperature, concentration of mineral and organic compounds) which can be experimentally evaluated in artificial enclosures, such as fermentors. Assuming

a representative value of $K_l a = 20\ h^{-1}$ estimated in unagitated flasks [1], then:

$$Q_{o_2}X = K_l a(C^* - \overline{C})$$
$$Q_{o_2}X = 11.6\ mg\ O_2/l/h$$

This value is definitly an overestimate as compared to a representative value of 5 mg O_2/l/h for oxygen production rate during day-time (A. Vaquer, personal communication). The latter exceeds significantly the respiration rate as demonstrated by oxygen sursaturation in the water layer. Therefore further experiments should be performed to evaluate *in situ* values of $K_l a$ for the flood layer over the rice field.

Considering the unresolved problems outlined by this study and the lack of available information in the literature, we may emphasize the need for further investigations on bacterial ecology of the rice field.

RÉSUMÉ

VARIATIONS À COURT TERME
DES PARAMÈTRES MICROBIOLOGIQUES ET PHYSICOCHIMIQUES
DANS L'EAU DE SUBMERSION D'UNE RIZIÈRE

Les variations nycthémérales du nombre de bactéries formant des colonies, du nombre total de bactéries et de certains paramètres physico-chimiques (température, pH, Eh, concentration d'oxygène, de carbonates, alcalinité totale) ont été étudiées dans l'eau de submersion d'une rizière. Les intervalles de confiance autour des moyennes des comptages ont été déterminés en fonction de l'hétérogénéité spatiale et de la fidélité des méthodes d'étalement sur boîte et de comptage par épifluorescence. L'agrégation bactérienne à l'échelle métrique et millimétrique a été mise en évidence, nécessitant la transformation des données pour les analyses statistiques des colonies sur milieu riche. Les variations significatives du nombre des bactéries formant des colonies ou de la biomasse bactérienne totale (évaluée en moyenne à 0.016 mg/l, poids sec) n'étaient pas directement corrélées avec les paramètres physicochimiques dans l'eau de rizière, indiquant ainsi l'influence supplémentaire de la prédation et des échanges avec les premiers millimètres du sédiment.

MOTS-CLÉS : Sol, Eau, Riz, Croissance bactérienne ; Variations à court terme, Physicochimie.

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